Translation of DE 199 19 625 A1 first part

Description

Every year approximately 20,000 patients in the USA alone die of the consequences of a heart valve dysfunction, and more than 60,000 patients are forced to have one or more heart valves operatively replaced due to an already recognized dysfunction. Possible replacements for the own heart valve are either mechanical or biological valve prostheses (xenografts). More infrequently, cryopreserved or glutaraldehyde-fixed homografts are used.

However, mechanical valve prostheses often lead to foreign body reactions with thromboembolic complications which are promoted in the heart by the changed flow conditions with the artificial heart valve. For this reason, lifelong anticoagulation of the affected patient is necessary, leading to a permanently increased danger of bleeding. Infections are a further, often life-threatening complication in patients with a mechanical heart valve.

Xenografts are usually pig valves treated with glutaraldehyde. Pig valve prostheses can be implemented in older patients with good results, but have a tendency to degenerate after only approximately 12 to 15 years, so that they are normally not an option for young people. Further, there is a higher risk of infection with pig valve prostheses as compared to the healthy heart. In addition, pig valves tend to calcify, making them unsuitable for use in children and young people who exhibit an increased calcium metabolism. Finally, they also represent tissue foreign to the body, which is recognized with a certain probability by the body's own immune system as foreign and can therefore trigger adverse immune processes.

Homografts are a third available option, i.e. fixed heart valves isolated from human donors. Although relatively resistant against infections, homografts also represent tissue foreign to the body, which leads with a certain probability to immune reactions. Further, just as with pig valve prostheses, homografts also tend to calcify and are therefore subject to substantial degeneration, which usually necessitates reoperation after 7 to 12 years. Further, homografts are extremely limited in their availability.

In addition to the disadvantages already discussed for valve prostheses used as valve replacement up to now, i.e. the triggering of immune reactions, the increased danger of infection, the danger of thromboembolic processes and the tendency to degenerate, a commonality among all valves known up to now is that they are made of inorganic material or fixed organic material and therefore lack important characteristics of a living matrix, for example the ability to undergo repair processes, reconfiguration or growth. From this it follows, among other things, that up to now regular reoperation has had to be accepted in pediatric valve patients. In addition to the risks inherent in any heart operation, the risk of morbidity and mortality increases with every reoperation since significant scarring occurs in the thorax

due to past operations.

There therefore exists an urgent need for a heart valve replacement which avoids the disadvantages discussed above. To this end, it has already been suggested to produce artificial heart valves by "tissue engineering". "Tissue engineering" relates to the development of "biohybrid" implants which grow into tissues

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or even into whole organ systems in the body. The production of biohybrid heart valves in the form of individual valve flaps has also already been described; however up to now the heart valve flaps have had the disadvantage that they have inadequate, insufficient connective tissue structures and therefore would not have been able to withstand the flow conditions present in the heart following dissolution of the biodegradable carrier structure. The problem of the invention is therefore to provide improved homologous heart valves as well as a method for their production.

According to the invention, the problem is solved by an in vitro process for the preparation of a homologous heart valve, comprising the following steps;

- providing a biologically degradable carrier (scaffold),
- populating the carrier with homologous fibroblasts and/or myofibroblasts to form a connective tissue matrix,
- populating the connective tissue matrix with endothelial cells
- placing, the connective; tissue matrix in a pulsatile flow chamber in which it can be subjected to increasing flow rates, and
- increasing the flow rates continually or discontinually

With the process according to the invention, homologous heart valves can be prepared, which on the basis of a pronounce core of connective tissue with natural collagen, elastin and glyycosamin content is suitable for implantation of a human patient. The processes for the preparation of the heart valve according to the invention as well as the heart valve prepared thereby will be further described in the following.

In the following description, the term "carrier" denotes an acellular structure which, as is further explained below, is made either of synthetic fibers or an acellular connective tissue scaffold. The term "matrix" denotes a connective tissue structure which, in addition to fibroblasts and myofibroblasts, contains typical components of an extracellular matrix, namely collagen, elastin and glycosaminoglycans. Structures denoted "matrix" typically contain the transitional phases between carrier and matrix are designated using the double designation “carrier/matrix”.

In performing the process according to the invention, first a biologically degradable carrier is provided. The carrier material should on the one hand be stable for a certain period of time in order to enable sufficient population or penetration with fibroblasts and/or myofibroblasts and formation of a

connective tissue matrix. On the other hand, complete hydrolytic dissolution of the carrier matrix should be possible within an acceptable time, which is ideally shorter than the time taken for the formation of the homologous valve prosthesis. It is preferred that the degradation begins after approximately 8 days; it should normally be completed in less than 3 months, preferably after 4 to 6 weeks. The carrier material is preferably a structure made of polymer fibers surrounding a porous polymer structure or an acellular biological tissue. Suitable synthetic polymers for this use include bio-erodible polymers, such as for example polyglycolic acid. (PGA), polylactic acid (PLA),

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polyhydroxyalkanoate (PHA) and poly-4-hydroxybutryrate (P4HB). These polymers can be used both in pure form as well as in mixtures of two or more of the substances indicated or mixtures of these substances with further biologically degradable polymers. A preferred embodiment uses a mixed polymer of 85% PGA and 15% PLA. Idearly the biologically degradable carrier is preformed in the form of the desired hard valve. Examples for possible hard valve forms are given in **figure** 1.

Biologically degradable carriers with a polymer density of approximately 40 to 120 mg/cm3 have proven useful. Below 40 mg/cm3 the polymer tissue is too labile. Above 120 mg/cm3 the tissue is too dense to allow penetration of fibroblasts within a reasonable length of time. In preferable embodiments the density of the biologically degradable carrier is 50 to 80 mg/cm3, especially preferred 70 mg/cm3. In the present invention a polymeric carrier of the company Albany International Research, Mensville, MA, USA with a density of approximately 70 mg/cm3 was used by the inventors with good results. The fibers of the carrier may have a diameter of 6 to 20 pm, preferably 10 to 18 pm. However, tissues with other fiber strengths are also conceivable which, on the one hand, must confer a certain stability on the carrier and on the other hand must also allow population and penetration of the carrier with fibroblasts or myofibroblasts. Pore sizes of 80-240 pm have proven to be favorable in porous (sponge-like) polymer forms. The pores can be achieved by the so-called "Salt-Leaching" technique, which is known to one of skill in the art.

Instead of a synthetic carrier as described above, it is conceivable to use an acellular connective tissue scaffold. For example, a pig valve could be transformed into an immunologically neutral tissue (Bader et al., Eur. J. -Cardiothorac. Surg. 14, 279, 1998), which could then be subsequently populated with homologous cells.

The biologically degradable carrier is first incubated with a population of fibroblasts. In using homologous fibroblasts and/or myofibroblasts, i.e. fibroblasts and/or myofibroblasts from a human, but not necessarily from the patient, attention should be paid to identical HLA-typing. Here, fibroblast populations can for example be obtained from peripheral blood vessels, as well as from arteries or also from veins. For this purpose, the arteria radialis of the lower arm presents itself in particular and is available in most cases for explantation without damage due to the double arterial supply: ofthe arm. Alternatively, vessel cells from blood vessels of the leg, for example the vena saphena can be obtained. Further, myofibroblasts and endothelial cells from bone marrow precursor cells or from pluripotent stem cells or genetically manipulated cells may be obtained.

For example, the cells can be obtained from vessel fragments in which, as described in Zünd et al. (Eur. J. of Cardiothorac. Surg. 13, 160, 1998), the tissue pieces are first cut into pieces of broken tissue and are incubated approximately 1 to 2 weeks under normal cell culture conditions (37°C, 5 002, 95% humidity), until the cells form a confluent cell layer on the bottom of the culture dish. Subsequently, they are subjected to multiple passages in order to obtain a cell culture free of extraneous tissue material. After two to three passages, the mixed cell populations may be purified by incubating them with a fluorescent label specific for endothelial cells (Dil-Ac-LDL, by Medical Technologies

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Inc., Stoughton, MA) and separated by means of flow cytometry (FACStar Plus, Becton Dickinson). Fluorescently labeled cells are endothelial cells, whereas non-labeled cells are fibroblasts and myofibroblasts. These are cultivated for a further two to three weeks and are subjected to two to four passages during this time in order to obtain a sufficient number of cells for the subsequent population of the carrier.

In the thus obtained fibroblast cultures fibroblasts and myofibroblasts are mixed. The ratio between the two populations is varying. It is preferred to use cultures, in which the fibroblast content is prevailing; particularly preferred is a fibroblast content of more than 75%.

A fibroblast/myofibroblast culture purified as described or any other purified fibroblast/myofibroblast culture may now be used for populating the polymer carrier. For this purpose, approximately 105 to 5 x 108 fibroblasts and/or myofibroblasts are used per square centimeter of the carrier surface. The term "surface" is in this case not the actual surface of the polymer, but rather the surface recognizable in a plane when viewing the carrier from above. Normally, the fibroblasts are given 60 to 90 min. time to adhere to the carrier. The medium above can then be subsequently removed and a fibroblast suspension can be added a further time. Ideally, however, one allows 2 to 36 hours, preferably 24 hours to elapse between the first and the second addition of fibroblast suspension.

In a preferred embodiment of the process according to the invention, fibroblasts and/or myofibroblasts are added a further 3 to 14 times, specially preferred 5 to 10 times to the carrier or the matrix gradually forming following the first addition of fibroblasts.

Under the conditions normally used for the cell growth of fibroblasts (for example 5% $C0_2$, incubation at 37°C, sterile medium) a solid connective tissue structure develops after approximately one to three weeks. In a preferred embodiment the structure is subsequently incubated with a pure endothelial cell suspension. Just as with the fibroblasts, the endothelial cells can also be enriched by FACS and subsequently expanded in multiple passages (preferably 3). For endothelial cells it is also preferred to repeat the population with approximately 10^5 to 5 x 10^8 endothelial cells each time, e.g., 3 to 14 times. In preferred embodiments, populating with endothelial cells is repeated 5 to 10 times. Two population steps should be separated by at least 60 min, preferably, however, by 2 to 24 hours. The step of populating with endothelial cells is however optional.

Preferably, human cells are used to populate the carrier. However, it is especially preferred to use autolgous fibroblasts and/or myofibroblasts and, optionally, endothelial cells. To this end, tissue, for

example from a vessel, is taken from the patient who is to undergo heart valve replacement. As already mentioned above, the arteria radialis as well as the vena saphena or bone marrow present themselves for this purpose. The use of autologous cells for the construction of the heart valve has the substantial advantage that the valve does not represent tissue foreign to the patient following implantation and, therefore, immune reactions against the artificial heart valve are virtually precluded.

Histological and immunohistochemical detection of a tissue with a superficial single layer of endothelial cells and a base structure of connective tissue is possible approximately 14 days after the optional addition of endothelial cells. This tissue is however useful for implantation into a human heart only in a limited

manner, since, due to its weak mechanical properties

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it would not be suitable for the flow conditions at the place of implantation.

In a further process step according to the invention, the pre-formed heart valve-analogous structure can now be placed in a pulsatile flow chamber, in which it can be subjected to increased flow rates. It was determined that the formation of a flow-resistant connective tissue matrix can be achieved by a slow adaptation of the flow rates.

For carrying out the method according to the invention a bioreactor is suitable. This bioreactor should be as compact as possible in order to be used in conventional cell incubators. The bioreactor should comprise a flow chamber, in which the flap-valve is located. The drive for the pumps of the fluid through the flow chamber should be located outside of the flow chamber. This is achieved in the bioreactor as used by the provision of an air chamber located neighboring the flow chamber, and separated from the flow chamber by a highly elastic membrane. By means of pulsating alteration of the pressure in the air chamber a transport of the fluid through the flow chamber is achieved. Instead of air also a liquid or another gas can be provided in the air chamber, which then can be generally designated as drive medium chamber. In this case this gas or a fluid therein has to be subjected to pulsating pressure changes. Air however can be handled particularly easily and therefore has proven to be particularly suitable for the operation of a bioreactor.

Due to the air chamber the drive for the transport of the fluid in the flow chamber can be constructed in a very compact manner and can also be located outside of the incubator. It is possible to thereby provide a separation between the part, which is directly acting on the flow chamber and the part which generates the pressure alterations in the air chamber, or drive medium chamber, respectively. Like this for example a respiratory pump can be located outside of the incubator, connected to the air chamber or drive medium chamber, respectively via a thin tube. Like this the design of the bioreactor and the drive itself can be adapted to the actual surroundings.

The pulsatile flow chamber for the method according to the invention is part of the bioreactor. A preferred embodiment of the bioreactor is in the following in detail described with respect to figures 2 and 3. It is clear that the bioreactor as well as the device including it is not limited for the use of the method according to the invention but can also be used independent therefrom.

The figures illustrate:

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